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Neuronal Control of Locomotion in *C. elegans* Is Modified by a Dominant Mutation in the GLR-1 Ionotropic Glutamate Receptor

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Summary

How simple neuronal circuits control behavior is not well understood at the molecular or genetic level. In *Caenorhabditis elegans*, foraging behavior consists of long, forward movements interrupted by brief reversals. To determine how this pattern is generated and regulated, we have developed novel perturbation techniques that allow us to depolarize selected neurons in vivo using the dominant glutamate receptor mutation identified in the Lurcher mouse. Transgenic worms that expressed a mutated *C. elegans* glutamate receptor in interneurons that control locomotion displayed a remarkable and unexpected change in their behavior—they rapidly alternated between forward and backward coordinated movement. Our findings suggest that the gating of movement reversals is controlled in a partially distributed fashion by a small subset of interneurons and that this gating is modified by sensory input.

Introduction

The functions of neural circuits depend upon the properties of the component neurons as well as the specifics of the neuronal interconnections. Yet, we still have limited insight into how information is encoded by an ensemble of neurons. This fundamental question can be addressed by the study of simple neural circuits. These circuits often control complex behaviors, including the generation of rhythmic motor behaviors. For example, in the lamprey it has been possible to define the neural networks that control locomotion (Grillner et al., 1995). Simple circuits have been the chief attraction of invertebrates (Marder, 1994), and a rich body of literature exists for the study of neural circuits in model systems, such as *Aplysia* (Frost and Kandel, 1995), *Manduca* (Christensen et al., 1993), leech (Lockery and Sejnowski, 1993), locust (Orchard et al., 1993), lobster (Harris-Warrick et al., 1992), and *Ascaris* (Stretton et al., 1985). Study of these simple circuits in invertebrates has mostly utilized biophysical techniques to measure the electrical potential of neurons and their synaptic transfer properties. These studies have led to a detailed description of the ionic currents underlying rhythmic neuronal behavior, enabling the modeling of neural networks (Grillner et al., 1995; Roberts et al., 1995). However, these types of studies have not yet been attempted in *C. elegans*.

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C. elegans has a simple nervous system yet is capable of complicated behaviors (Bargmann, 1993; Bargmann and Kaplan, 1998). One advantage of *C. elegans* is that defined processes and behaviors can be analyzed by genetic perturbation (Bargmann, 1993; Bargmann and Kaplan, 1998), an approach that complements biophysical studies. To determine how movement is controlled in the soil nematode *C. elegans*, we have developed a novel perturbation technique that permits the chronic activation of defined neuronal circuits.

In the laboratory environment, the worm exhibits a characteristic pattern of movement on agar plates that is biased toward moving forward and is interrupted by periodic brief reversals. The neuronal circuit that controls locomotion has been described by Chalfie, White, and coworkers (Chalfie et al., 1985; White et al., 1986). In particular, they identified five pairs of interneurons that are required for the control of coordinated movement (Figure 1A). These have been termed the command interneurons. Laser ablation studies have dissected this circuit into two parts: neurons PVC and AVB are primarily required for forward movement, and neurons AVA, AVD, and AVE for backward movement (Chalfie et al., 1985).

Worms can be directed to move either backward or forward by tactile stimulation. *C. elegans* has specialized receptors that can sense touch or vibration (Chalfie et al., 1985; Wicks and Rankin, 1995). These sensory neurons make synaptic or gap junction contacts with the command interneurons (Figure 1A). When a worm receives tactile stimulation to its body, the basic locomotory pattern is interrupted, and the worm initiates an avoidance response by either reversing direction or accelerating its movement. This escape response is also mediated by the command interneurons (Chalfie et al., 1985).

Another escape response is mediated by the sensory neuron ASH, which makes synaptic contacts with the command interneurons. This polymodal sensory neuron detects mechanical stimuli to the nose of the worm, the local osmotic strength of its environment, and volatile repellants. Worms in which ASH is ablated by laser do not move backward when they encounter either a mechanical stimulus, such as a hair in their path, or hyperosmotic conditions (Bargmann et al., 1990; Kaplan and Horvitz, 1993; Troemel et al., 1995). ASH is likely to signal touch to the command interneurons via glutamatergic synapses (Lee et al., 1999). Mutations in *glr-1*, a gene encoding a non-NMDA glutamate receptor subtype, are associated with disrupted mechanosensation mediated by the sensory neuron ASH (Hart et al., 1995; Maricq et al., 1995). Worms with mutations in *glr-1* have a disrupted escape response to mechanical stimuli to the nose. Interestingly, the osmotic avoidance response is unimpaired. *glr-1* is expressed exclusively in a subset of neurons, including the interneurons AVA, AVD, and AVE (the backward command interneurons), and AVB and PVC (the forward command interneurons; Figure 1A). These interneurons receive sensory information either directly or indirectly from a wide variety of sensory neurons and interneurons. In addition, the forward and

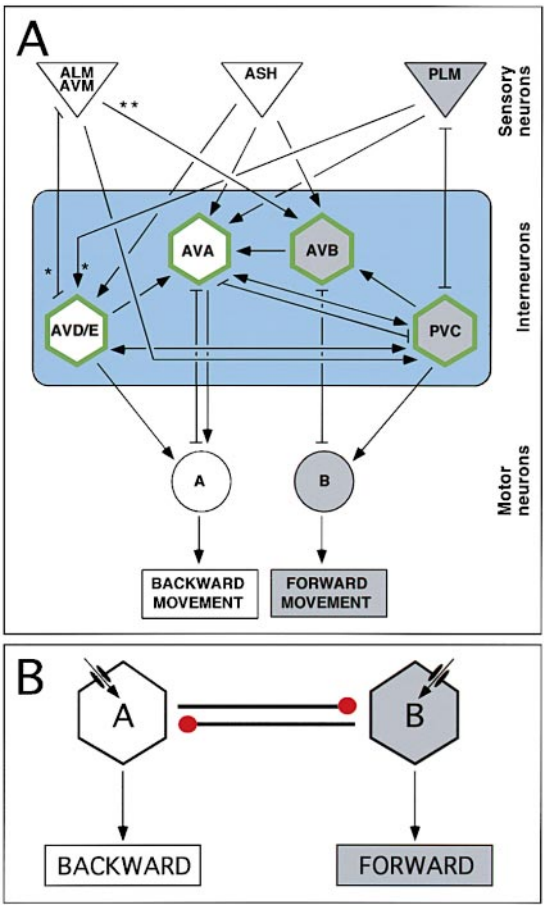

Figure 1. Schematic of the Neural Circuitry that Controls the Modulation and Coordination of Locomotion in *C. elegans*

(A) Locomotory control circuitry. The assignment of function of the neurons in this circuit was primarily based on electron microscopic studies that led to the reconstruction of the worm's nervous system and on subsequent laser ablation studies (Chalfie et al., 1985; White et al., 1986). Neurons that are required for the initiation or control of forward (AVB, PVC) and backward (AVA, AVD/AVE) movement are shown in gray and white, respectively. Those that have been shown to express GLR-1 are outlined in green. The command interneurons make gap junction contacts with subsets of motor neurons (A class, B class) that contact muscles required for forward or backward movement. Specialized sensory neurons that detect touch to the body (anterior body touch: ALM, AVM; posterior body touch: PLM) provide input to neurons in both the forward and backward circuits in a characteristic fashion. For example, ALM has gap junction contacts (indicated by ⊣) with AVD, presumably to rapidly initiate backward movement; it also has chemical synapses (indicated by arrows) with PVC that are hypothesized to be inhibitory and to suppress coincident forward movement (Chalfie et al., 1985; Lee et al., 1999). The polymodal sensory neuron ASH also makes synaptic contacts with the command interneurons. Putative chemical and electrical synaptic connections have been assigned on the basis of serial electron microscopic sections of the *C. elegans* nervous system (White et al., 1986). Asterisk, AVD only; double asterisk, AVM only.

(B) A simple pattern generator. The backward and forward control elements can be modeled as two neurons that have mutually inhibitory connections with one another (in red) and excitatory connections to the motor neurons that control locomotion. This circuit can be made to oscillate between the forward and backward states. The dwell time in each state would be determined by the strength of the inhibitory synaptic inputs and the sum of the excitatory inputs. The dwell time in either state would be shortened by the injection of additional depolarizing current. The mutant GLR-1(A/T) receptor would act to inject current into both neuronal elements and thereby increase the rate of switching between forward and backward states.

backward command interneurons make synaptic connections with one another. Somehow, the information is integrated and the output directed to distinct subsets of motor neurons that propel the worm either forward or backward (Figure 1).

To understand the movement responses to various sensory inputs, one must first understand the control of locomotion. How is the periodicity of directional movement controlled by the nervous system of *C. elegans*? One hypothesis is that the periodicity is intrinsic to the command interneuron circuitry, that is, it is a function of both the membrane properties of the component neurons and the connections between the neurons. Alternatively, timing behavior could be controlled by neurons external to this circuit. For example, periodic depolarizations of the touch-responsive sensory neurons may regulate the timing of forward and backward locomotion. To distinguish between these possibilities and to determine the properties of the locomotory control circuit, we have selectively depolarized subsets of neurons using a modified glutamate receptor.

Our approach to depolarizing selected neurons in *C. elegans* relies on the targeted expression of an activated glutamate receptor. Glutamate receptors are essential for the function of all nervous systems, and they play pivotal roles in synaptic transmission, synaptic plasticity, and neuronal disease in the mammalian nervous system (Nakanishi, 1992; Seeburg, 1993; Choi, 1994; Hollmann and Heinemann, 1994; Scheetz and Constantine-Paton, 1994). Our strategy was motivated by the recent identification of the underlying defect in the Lurcher mouse (Phillips, 1960; Norman et al., 1995; Cheng and Heintz, 1997); it is a missense mutation in the δ2 subtype of the ionotropic glutamate receptor (Lomeli et al., 1993) that results in an alanine-to-threonine (A/T) change in a highly conserved region of transmembrane domain III (TM III) (Zuo et al., 1997). Ionotropic glutamate receptors are oligomeric transmembrane proteins that form an ion channel that can be gated by the binding of extracellular glutamate. The mutated alanine is strongly conserved and is found in essentially all known ionotropic glutamate receptors, including the GLR-1 glutamate receptor in *C. elegans* (Figure 2A). Lurcher mice display an ataxic gait secondary to cerebellar damage. The Lurcher mutation may change the gating properties of the receptor, resulting in a chronically open channel that is independent of the presence of ligand. When cRNA encoding the mutant receptor was expressed in *Xenopus* oocytes, a large increase in leakage current was observed, consistent with a chronically activated receptor (Zuo et al., 1997). In Lurcher mice, the Purkinje neurons are chronically depolarized, dysfunctional, and prone to apoptotic death (Norman et al., 1995; Cheng and Heintz, 1997).

Here, we show that a dominantly active *C. elegans* glutamate receptor can be generated by introducing the Lurcher amino acid change into the GLR-1 glutamate

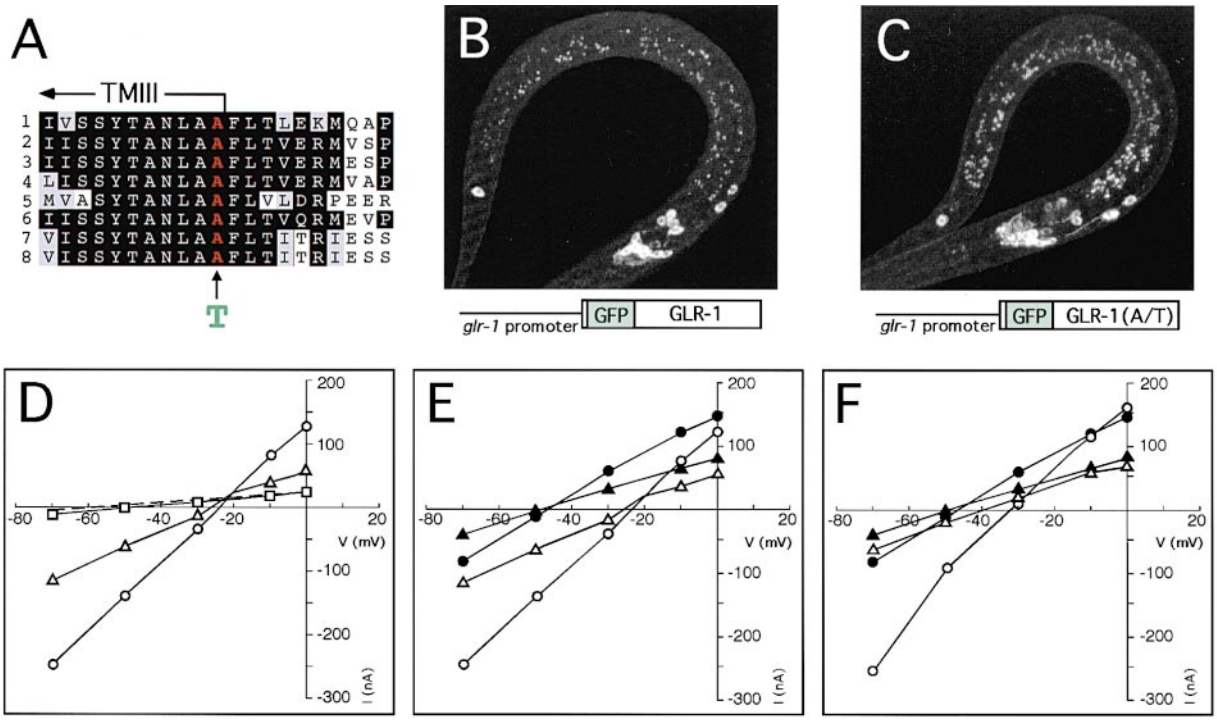

Figure 2. GLR-1(A/T) Forms a Nontoxic, Constitutively Open Channel that Can Be Expressed in the Command Interneurons

(A) Alignment of the C-terminal region of TM III of ionotropic glutamate receptors. Shown are representative receptors of the non-NMDA and NMDA classes from *C. elegans* GLR-1 (1), rat GluR1 (2), rat GluR5 (3), *Drosophila* GluR1 (4), rat NR1 (5), rat Ka1 (6), rat δ2 (7), and mouse δ2 (8). The conserved alanine found in TM III is shown in red. This residue is mutated to a threonine (shown in green) in the mouse Lurcher strains *Lc* and *Lc*^{J}. We have engineered an identical substitution into *C. elegans* GLR-1(A/T).

(B and C) GFP expression in transgenic strains. Under control of the *glr-1* promoter, larval transgenic strains expressed chimeric proteins that contained GFP fused to the N terminus of wild-type GLR-1 (B) or GLR-1(A/T) (C). Images acquired with confocal microscopy show that transgenic strains expressed the GLR-1(A/T)::GFP fusion protein in the appropriate neurons and that these neurons show no evidence of gross morphological abnormalities.

(D–F) GLR-1(A/T) forms a ligand-independent leaky channel in *Xenopus* oocytes. cRNA was prepared in vitro from cDNA clones encoding GLR-1, GLR-1(A/T), or GLR-1(A/T;Q/R) and was injected into *Xenopus* oocytes. After 3 days, transmembrane currents were recorded by standard two-electrode voltage-clamp techniques (Marcus-Sekura and Hitchcock, 1987).

(D) I–V relations obtained from oocytes bathed in standard oocyte ringer solution that contained 2 mM Ca^{2+} (OR2). Uninjected oocytes (dashed line, n = 7) were indistinguishable from oocytes injected with cRNA encoding GLR-1 (open squares, n = 12); both had a small leakage conductance, and the current reversed direction from inward to outward near −50 mV (reversal potential). In contrast, oocytes injected with cRNAs encoding either singly mutant (open circles, GLR-1[A/T], n = 11) or double mutant (open triangles, GLR-1[A/T;Q/R], n = 11) receptors were significantly leakier than control oocytes. In addition, the reversal potential has shifted right to a more depolarized level, indicating the presence of a membrane conductance with a reversal potential that is near −20 mV.

(E) GLR-1(A/T) is primarily permeant to cations. Shown are I–V relations obtained from oocytes (D) that were switched to a salt solution in which Na^{+} (open symbols) was replaced by the large organic cation NMDG (closed symbols). GLR-1(A/T)-injected oocytes (circles) were leakier than GLR-1(A/T;Q/R)-injected oocytes (triangles), both before and after the switch to NMDG, which was accompanied by a leftward shift of the reversal potential to a more hyperpolarized level.

(F) GLR-1(A/T;Q/R) is less permeant to the divalent cation Ca^{2+}. Shown are I–V relations obtained from oocytes in (E) that were switched to a salt solution in which the NMDG containing 2 mM Ca^{2+} solution (closed symbols) was changed to an isoosmotic NMDG solution containing 50 mM Ca^{2+} (open symbols). GLR-1(A/T)-injected oocytes (circles) became significantly leakier after the switch to the high-Ca^{2+} NMDG solution. This conductance increase was accompanied by a rightward shift of the reversal potential to a more depolarized level, indicating that GLR-1(A/T) is permeant to Ca^{2+}. In contrast, oocytes that expressed GLR-1(A/T; Q/R) had only a small change in conductance and reversal potential when changed to the solution containing 50 mM Ca^{2+}.

receptor. When this modified receptor, GLR-1(A/T), was expressed in *Xenopus* oocytes, it formed a leaky channel. Transgenic worms (*akIs9*) that expressed this altered receptor in the command interneurons showed a dramatic and unexpected change in their behavior, rapidly alternating between forward and backward movement. The simplified wiring diagram of the nervous system (Figure 1A) shows that the command interneurons have reciprocal synaptic contacts as well as gap junction contacts. This arrangement suggests that the forward and backward command interneurons may function as a bistable switch (Chalfie et al., 1985) that is mediated by the synaptic contacts between the command interneurons. Our results provide evidence for this hypothesis and suggest that the frequency of switching is mediated by depolarization.

Using a combination of genetic and laser ablation techniques, we show that the command interneurons function to gate forward and backward movement and that the intrinsic activity of this circuit can be modified

by sensory input. We also show that the GLR-1(A/T) perturbation technique is generally applicable in that it can be used to activate neurons that do not normally express glutamate receptors.

Results

GLR-1(A/T) Forms a Leaky Channel

To test whether GLR-1 could be chronically activated and function to depolarize neurons, we used standard methods for site-directed mutagenesis to introduce one or two amino acid changes into a *glr-1* cDNA clone. The first mutation changed the conserved alanine in TM III into threonine; this mutation is equivalent to that found in the δ2 glutamate receptor subunit of the Lurcher mouse (Figure 2A) (Zuo et al., 1997). The second mutation was a glutamine-to-arginine (Q/R) change in TM II that is known to affect both the current–voltage (I–V) relation and ion permeation of vertebrate non-NMDA receptors (Hume et al., 1991; Burnashev et al., 1992). The proteins encoded by these mutated *glr-1* cDNAs are GLR-1(A/T) and GLR-1(A/T;Q/R), respectively.

We injected into *Xenopus* oocytes RNA transcripts (cRNA) generated in vitro from wild-type and mutated *glr-1* cDNA. For oocytes injected with wild-type *glr-1* cRNA, no detectable currents were evoked with up to millimolar concentrations of glutamate (data not shown), and the I–V relation in the absence of glutamate was indistinguishable from that of control oocytes (Figure 2D). In a few cases, we observed small, nA-sized currents in response to 30 mM glutamate (data not shown). In contrast, oocytes injected with cRNA encoding the Lurcher variant, GLR-1(A/T), had a 10-fold higher membrane conductance in the absence of agonist, suggesting that GLR-1(A/T) forms an ion channel in the oocyte membrane. To test whether the increased conductance was specific to the introduction of GLR-1(A/T), oocytes were injected with cRNA encoding GLR-1(A/T;Q/R). The conductance of these oocytes was at a value intermediate to that of control- and GLR-1(A/T)-injected oocytes. In mammals, the Q/R mutation changes an amino acid that is a key determinant of calcium permeation through the channel (Sommer et al., 1991; Brusa et al., 1995; Seeburg, 1996). Because the addition of a second-site Q/R mutation decreased the conductance, we presume that the increased membrane conductance of oocytes that express GLR-1(A/T) is pore mediated and not due to nonspecific membrane changes. Thus, in *Xenopus* oocytes, GLR-1(A/T) forms a homomeric receptor that has a constitutively open channel in the absence of ligand.

All known vertebrate ionotropic glutamate receptors are cation permeant. In *C. elegans*, a second family of glutamate-gated anion-permeant channels has been recently discovered, but these receptors are not related by sequence to *glr-1* or vertebrate glutamate receptors (Cully et al., 1994; Dent et al., 1997). To determine whether GLR-1(A/T) is primarily permeant to cations or anions, we varied the concentration of external cations and examined the I–V relations of oocytes that express GLR-1(A/T) or GLR-1(A/T;Q/R). When all external Na^+ was replaced by the larger, less permeant cation N-methyl D-glucamine (NMDG), the potential at which no net current flowed (reversal potential) shifted by nearly 25 mV, from −23.5 mV to −48.2 mV (Figure 2E). This result suggests that GLR-1(A/T) is permeant to both Na^+ and K^+ and not appreciably permeant to anions. Removing external Na^+, leaving K^+ as the dominant ionic species, shifted the reversal potential to the left—closer to the calculated Nernst potential for K^+ (Figure 2E). This is consistent with a conductance that is permeant to both cations. A similar shift was observed for GLR-1(A/T;Q/R), from −28 mV to −48 mV, suggesting that this doubly modified channel is also significantly permeant to both K^+ and Na^+.

GLR-1(A/T) is also permeant to the divalent cation Ca^{2+}. Oocytes were bathed in a solution in which the Na^+ was replaced with NMDG. When the external Ca^{2+} concentration was raised from 2 mM to 50 mM, the I–V relation shifted ∼14 mV in a depolarizing direction, suggesting that GLR-1(A/T) was significantly permeant to Ca^{2+} (Figure 2F). The Q/R mutation is known to affect the Ca^{2+} conductance of non-NMDA type glutamate receptors (Hume et al., 1991; Burnashev et al., 1992) and also affects the Ca^{2+} conductance of GLR-1(A/T). When the same solution changes were applied to oocytes that expressed the GLR-1(A/T;Q/R) variant, the reversal potential shifted right 10 mV, again consistent with GLR-1(A/T) being Ca^{2+} permeant and the Q/R mutation decreasing the Ca^{2+} conductance.

GLR-1(A/T) Is Nontoxic When Expressed In Vivo

To perturb neuronal function in *C. elegans*, we expressed GLR-1(A/T) in transgenic strains. We introduced the same Lurcher A/T mutation into a genomic *glr-1* clone, generating a gain-of-function allele that encodes GLR-1(A/T). In addition, we generated a Q/R allele that encodes GLR-1(Q/R), and the double mutant allele that encodes GLR-1(A/T;Q/R). Variants of the GLR-1 protein were expressed in transgenic strains using the *glr-1* promoter. To determine the expression pattern of the modified GLR-1 proteins, we examined the fluorescence pattern in transgenic strains that expressed either an N-terminal *glr-1::GLR-1::GFP* chimeric fusion protein (*akEx45*) or a modified version of this fusion protein (*glr-1::GLR-1[A/T]::GFP*) that included the A/T lurcher mutation (*akEx55*). Expression of the GLR-1::GFP fusion protein was limited to a subset of neurons, including the command interneurons (Figure 2B). Transgenic strains that expressed *glr-1::GLR-1(A/T)::GFP* exhibited green fluorescent protein (GFP) fluorescence in the same neurons. There was no alteration in the expression pattern or in the apparent level of expression, suggesting that the A/T mutation did not affect protein expression, general distribution, or stability (Figure 2C). Expression was not detected in transgenic worms injected with a promoterless construct (data not shown). The behavioral consequences associated with the A/T mutation (see below) did not differ between strains that expressed either the chimeric GLR-1(A/T)::GFP protein or the GLR-1(A/T) protein (data not shown). In mice, the Lurcher mutation causes eventual neuronal death. However, in transgenic worm strains that expressed GLR-1(A/T), neuronal number was not affected, suggesting that neuronal development was not altered by expression of GLR-1(A/T) and that the altered proteins were not obviously toxic or injurious to the cells.

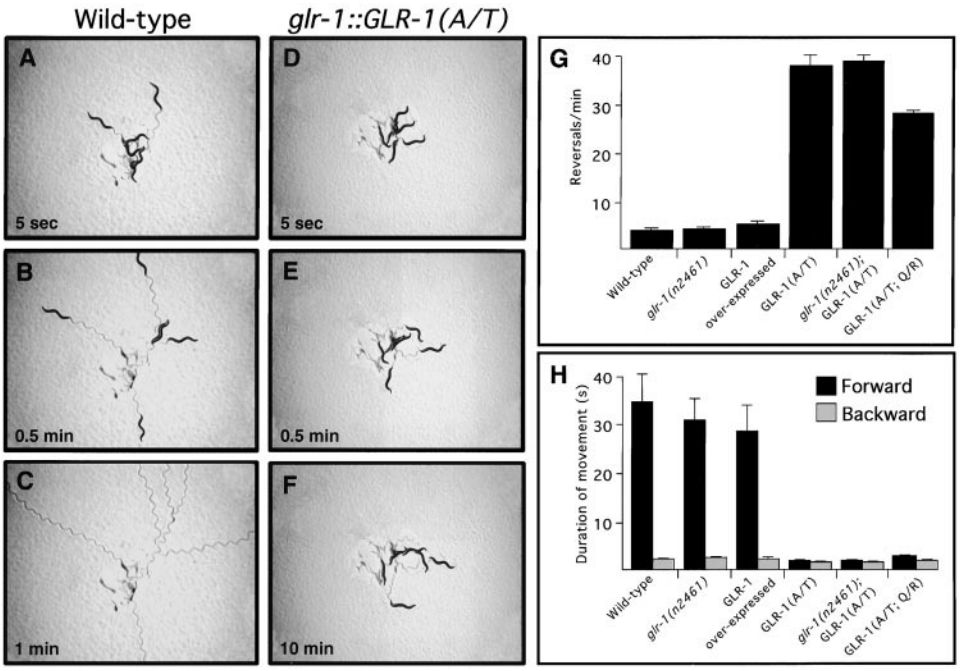

Figure 3. *glr-1::GLR-1(A/T)* Worms Exhibit a Greatly Increased Frequency of Movement Reversals

Wild-type worms (A–C) and *glr-1::GLR-1(A/T)* worms (D–F) were placed on separate agar plates covered by a thin lawn of bacteria. Immediately after being placed at their starting positions, wild-type worms began to disperse (A). Within 30 s, the same worms were almost out of the field of view (B), and by 1 min they had left the field of view (C). In contrast, *glr-1::GLR-1(A/T)* worms (D–F) took much longer to disperse. *glr-1::GLR-1(A/T)* worms remained at their starting positions immediately after being placed on the agar plate (D). After 30 s (E), or even after 10 min (F), the worms had not moved more than a few body lengths. *glr-1::GLR-1(A/T)* worms moved in a normal sinusoidal motion and did not appear uncoordinated or sluggish.

(G and H) Individual worms were placed on food-free agar plates, and the duration of each forward and backward movement was recorded. Wild-type worms moved forward at 0.18 mm per second ($\pm$ 0.01 s, n = 10), whereas *glr-1::GLR-1(A/T)* worms moved forward at 0.21 mm per second ($\pm$ 0.01 s, n = 10). Each transition in direction of movement is called a reversal. Wild-type worms, *glr-1(n2461)* null mutants, and transgenic worms that overexpressed the wild-type *glr-1* gene (GLR-1 overexpressed) reversed direction $\sim$4 times per minute, whereas *glr-1::GLR-1(A/T)* worms reversed $\sim$38 times per minute (G). This behavior was independent of the wild-type GLR-1 protein (*glr-1[n2461];GLR-1 [A/T]*). The frequency of reversals could be further modified by introducing a second-site mutation that caused a Q/R change in the pore-lining region of TM II. Worms that expressed GLR-1 with both the A/T and Q/R change (GLR-1[A/T; Q/R]) showed a reduction in the number of reversals per minute compared with *glr-1::GLR-1(A/T)* worms. The primary reason for the increased number of reversals in *glr-1::GLR-1 (A/T)* worms was that they had a greatly decreased duration of forward motion (H). Wild-type worms, *glr-1(n2461)* mutants, and worms that overexpressed GLR-1 traveled forward for an average of 30–35 s before they reversed direction and moved backward for 2.5 s. In contrast, the duration of both forward ($\pm$ 0.12 s, $p < 0.001$) and backward motion in *glr-1::GLR-1(A/T)* worms was $\sim$1.7 s. The duration of forward movement could be increased to 2.5 s by introducing the second-site mutation (Q/R) in GLR-1(A/T), as seen in GLR-1(A/T;Q/R) worms ($\pm$ 0.12 s, $p < 0.001$). Similar behavior was observed in three additional strains that expressed *glr-1::GLR-1(A/T;Q/R)*. The average forward durations for these three strains were 2.5 s ($\pm$ 0.15 s, $p < 0.02$); 2.6 s ($\pm$ 0.09 s, $p < 0.002$); and 2.5 s ($\pm$ 0.13 s, $p < 0.02$). In all cases, n = 10.

Transgenic Worms that Overexpress GLR-1(A/T) Exhibit a Drastically Modified Pattern of Locomotion

Compared with wild-type worms, which move forward for a long duration while foraging, *glr-1::GLR-1(A/T)* worms (an integrated transgenic strain, *akIs9*, that expressed GLR-1(A/T) under control of the *glr-1* promoter), moved forward for only a short duration before reversing direction. This change in locomotory behavior dramatically impaired their ability to move long distances. Within seconds of being placed on an agar plate, wild-type worms began to disperse (Figures 3A and 3B). By 1 min, the worms had moved many body lengths and were out of the field of view (Figure 3C). *glr-1::GLR-1(A/T)* worms did not disperse after being placed on an agar plate (Figures 3D and 3E) and even after 10 min were located within a few body lengths of their original position (Figure 3F). This was not a consequence of a reduced velocity of movement. Although *glr-1::GLR-1(A/T)* worms moved forward at near normal speed, within a few seconds they reversed direction. Consequently, they were incapable of effective movement. *glr-1::GLR-1(A/T)* worms had an otherwise normal appearance, although they were slightly smaller than wild-type worms.

On a food-free plate, wild-type worms reversed direction about 4 times per minute, as did worms mutant for *glr-1(n2461)* (Hart et al., 1995) or transgenic worms that overexpressed GLR-1 under control of the *glr-1* promoter (*akEx14*) (Figure 3G). In contrast, *glr-1::GLR-1(A/T)* worms reversed direction about 38 times per minute. A second transgenic strain that overexpressed GLR-1 (A/T) reversed 40 times per minute (data not shown). This hyperreversal behavior was dominant (Figure 4).

The increased number of reversals in *glr-1::GLR-1(A/T)*

worms was a consequence of the dramatic reduction in forward transit time (Figure 3H). Wild-type worms and *glr-1::GLR-1* transgenic worms (*akEx14*) were indistinguishable, both for reversal number and forward transit time, suggesting that the phenotype was not simply due to increased expression of the GLR-1 receptor. A *glr-1* null mutation (*n2461*) (Hart et al., 1995) did not affect the duration of forward or backward movement. In contrast, compared with wild-type worms, the forward duration of *glr-1::GLR-1(A/T)* worms was reduced 20-fold. When *glr-1::GLR-1(A/T)* was expressed in a *glr-1* null background (*n2461*), the movement did not change appreciably.

To test whether the phenotype associated with expression of *glr-1::GLR-1(A/T)* was a consequence of an increased or unregulated channel conductance, we introduced a second-site mutation by changing glutamine to arginine at the Q/R site of the genomic clones encoding GLR-1 and GLR-1(A/T). This modification should alter only the permeability of the channel. Consistent with a decreased channel conductance (Figure 2D), transgenic worms that overexpressed the double mutant GLR-1(A/T;Q/R) (*akEx75*) had fewer reversals than *glr-1::GLR-1(A/T)* worms ($\sim$75%; Figure 3G). The second-site Q/R mutation modified both the forward and backward durations, increasing the average forward time by almost 50%. This result was expected since this mutation decreased, rather than eliminated, channel conductance when expressed in oocytes (Figure 2D).

glr-1::GLR-1(A/T) Worms Hyperreverse under Many Conditions

Typically, foraging worms slow their rate of movement and their reversal frequency when they encounter food. When on food, wild-type worms moved forward on average for 67 s ($\pm$ 8 s, $n = 10$, $p < 0.01$) and backward for 4.1 s ($\pm$ 0.5 s, $n = 10$, $p < 0.01$), demonstrating that food significantly increases the duration of both forward and backward movement. Food had a smaller but statistically significant effect on the hyperreversal phenotype of *glr-1::GLR-1(A/T)* worms. When on food, *glr-1::GLR-1 (A/T)* worms moved forward on average for 2.5 s ($\pm$ 0.15 s, $n = 9$, $p < 0.001$) and backward for 1.8 s ($\pm$ 0.1 s), indicating that food weakly suppressed the hyperreversal movements. The hyperreversal phenotype was observed in both hermaphrodites and males, and at all developmental stages, including the long-lived dauer stage. Dauer worms are a special, structurally unique larval stage that develops in response to harsh environmental conditions. Many tissues and behaviors are altered in dauers, including locomotion (Thomas, 1993; Riddle and Albert, 1997). Most wild-type dauers lie motionless when undisturbed and move rapidly after being jostled. For wild-type dauer worms, the average duration of forward and backward movement after being transferred to a food-free assay plate was 117 s ($\pm$ 18.4 s, $n = 10$, $p < 0.001$) and 2.4 s ($\pm$ 0.2 s, $n = 10$), respectively. In contrast, most *glr-1::GLR-1(A/T)* dauers constantly moved backward and forward, although not as often as nondauer *glr-1::GLR-1(A/T)* worms. For *glr-1::GLR-1 (A/T)* dauers, the average duration of forward and backward movement on food-free assay plates was 6.6 s ($\pm$ 0.9 s, $n = 10$, $p < 0.001$) and 1.4 s ($\pm$ 0.14 s, $n = 10$), respectively. These results suggest that the command circuitry is affected by the dauer state, and consequently, the effects of GLR-1(A/T) are modified. Interestingly, some *glr-1::GLR-1(A/T)* dauers were stationary when observed undisturbed on old, starved-out plates. When prodded into movement, they resumed their hyperreversal movements.

The Hyperreversal Phenotype Does Not Require Mechanosensory Input

The dramatic increase in number of reversals may reflect a direct influence on the command interneuron circuitry or, alternatively, may involve a contribution of retrograde and reentrant signaling via gap junctions to the sensory neurons ALM and PLM (Figure 1A). For example, activation of AVD by GLR-1(A/T) could activate ALM via gap junctions, which in turn make synapses with PVC. The two extreme possibilities are that either the command interneurons function as an intrinsic gating circuit or that command interneurons act simply as a relay station for sensory input. To distinguish between these two alternatives, we undertook an analysis of the behavior of *glr-1::GLR-1(A/T)* worms in various genetic backgrounds that affect the mechanosensory neurons ALM and PLM. These included mutations that affect the birth (*unc-86*) (Chalfie et al., 1981; Finney and Ruvkun, 1990), function (*mec-3*) (Way and Chalfie, 1988), or viability (*mec-4*) (Driscoll and Chalfie, 1991) of these sensory neurons. In general, the mutants did not move as actively on food as wild-type worms (Figure 4). However, each of the single mutants had fairly normal durations of forward and backward movement. This suggested that the command interneurons can establish a pattern of locomotion that is independent of mechanosensory input from the body wall touch receptors. A partial test of this hypothesis was provided by an examination of *glr-1::GLR-1(A/T)* worms; the hyperreversal behavior of *glr-1::GLR-1(A/T)* worms was not influenced by the *mec-3(e1338)*, *mec-4 (e1611dm)*, or *unc-86(n846)* mutations (Figure 4). The *glr-1::GLR-1(A/T)* phenotype was dominant, did not require input from the sensory neurons, and acted to modify the activity of the command interneuron local circuitry.

The hyperreversal phenotype might depend on other sensory input. To examine this possibility, we examined *glr-1::GLR-1(A/T)* worms that also had mutations in the *eat-4* gene. The *eat-4* gene encodes a homolog of a mammalian, brain-specific Na^+-dependent inorganic phosphate cotransporter that is believed to be required for glutamate uptake and glutamatergic neurotransmission (Lee et al., 1999). EAT-4 is expressed in the body wall touch cells ALM, AVM, and PLM, and touch sensitivity is disrupted in *eat-4* mutants—presumably as a result of defective glutamatergic neurotransmission. In addition, EAT-4 is expressed in the polymodal sensory neuron ASH, but it is not expressed in the command interneurons. *eat-4* mutants are defective in detecting osmotic gradients and in their backing response to nose touch or volatile repellants (Hart et al., 1999). *eat-4(ad572)* worms moved forward 2-fold longer than wild-type worms before reversing direction (Figure 4). In contrast, *glr-1::GLR-1(A/T);eat-4(ad572)* worms were quite similar in their movement to *glr-1::GLR-1(A/T)* worms. Thus, sensory input dependent on the function of EAT-4 was not required for *glr-1::GLR-1(A/T)* worms to hyperreverse.

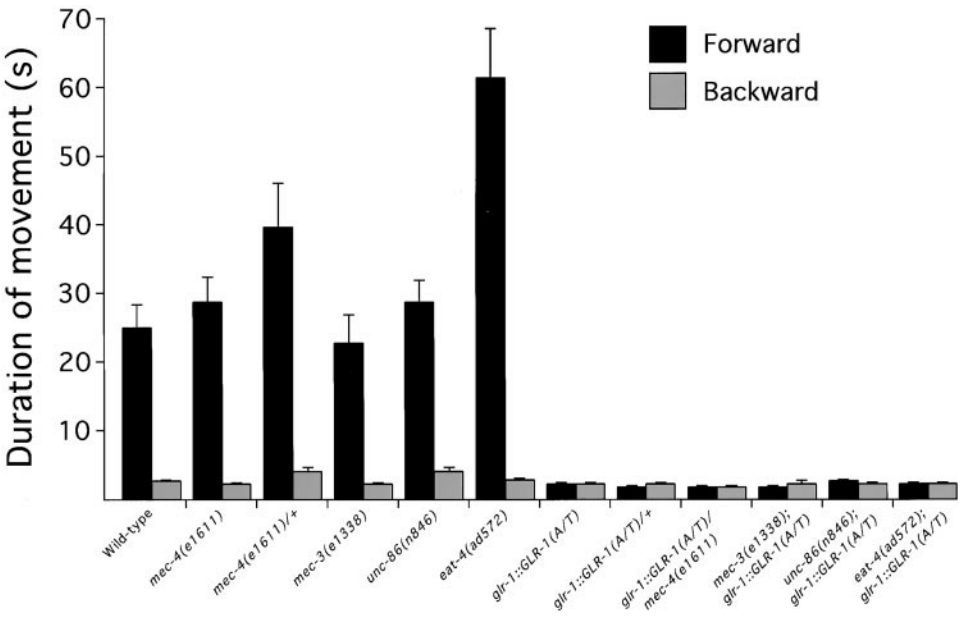

Figure 4. The Pattern of Locomotion in GLR-1(A/T) Worms Is Independent of Touch Cell Input

Mutant worms that had defects in mechanosensation due to defective differentiation of the sensory touch cells (*mec-3[e1338]*), degenerative death of the touch cells (*mec-4[e1611dm]*), lineage defects that lead to an absence of the touch cells (*unc-86[n846]*), or defective synaptic transmission (*eat-4[ad572]*) all had fairly normal locomotion. On food-free assay plates, *mec-4* and *mec-3* mutants moved at near normal velocity compared with wild-type worms (0.19 ± 0.02 mm per second and 0.16 ± 0.03 mm per second, respectively). Although *unc-86* mutants moved continuously on food-free plates, they did show a significant decrease in velocity (0.09 ± 0.01 mm per second, $n = 5$, $p < 0.001$). When *glr-1::GLR-1(A/T)* worms were crossed into these mutant strains, a dramatic change in locomotory behavior was observed: all of the worms showed a decrease in forward duration and a pattern of locomotion that was nearly identical to those of *glr-1::GLR-1(A/T)* worms. *eat-4(ad572)* or *eat-4(ky5)* worms moved forward an average of 62 s (± 7.4 s, $n = 18$, $p < 0.001$) or 120.2 s (± 16.5 s, $n = 7$, $p < 0.001$), respectively, whereas *glr-1::GLR-1(A/T);eat-4(ad572)* worms moved forward for an average of 2.2 s (± 0.1 s, $n = 10$).

Expression of the Human Caspase ICE under Control of the *glr-1* Promoter Causes an Apoptotic Death of Neurons

Under control of the *glr-1* promoter, GLR-1(A/T) is expressed in the command interneurons as well as in a number of other interneurons and motor neurons (Hart et al., 1995; Maricq et al., 1995). This expression is accompanied by a hyperreversal phenotype. Which of these neurons contribute to the control of movement, and in which neurons is expression of GLR-1(A/T) required for the hyperreversal phenotype? To determine whether neurons other than the command interneurons significantly contribute to the control of movement, we generated two strains of transgenic worms that lacked either all of the neurons that normally express GLR-1 or that lacked only a small subset of these neurons. Our approach, similar to that of Shaham and Horvitz (1996), used neuronal specific promoters to drive the expression of a caspase that would induce an apoptotic death of the neurons. Neuronal expression of a cDNA encoding human interleukin-1β-converting enzyme (ICE) (Cerretti et al., 1992; Thornberry et al., 1992), a caspase, results in an apoptotic death of the neurons (Figure 5). In a *ced-1(e1735)* background, in which engulfment of dead cells is inhibited (Hedgecock et al., 1983), refractile apoptotic bodies can be clearly identified (Figure 5B). In a transgenic strain that expressed ICE and GFP in the same neurons, apoptotic death of the neurons developed after the L1 larval stage, and by the early adult stage almost all GFP expression was eliminated (Table 1). GFP expression in the ICE-expressing transgenic strain correlates with the behavioral criteria. Loss of forward and backward command interneurons is associated with complete loss of touch sensitivity (Chalfie et al., 1985). Worms that were touch insensitive did not show GFP expression (Table 1), nor could GLR-1 immunoreactivity be detected (Figure 5).

Despite the neuronal death, transgenic strains that expressed *glr-1::ICE* (*kyIs36*) could still move in a sinusoidal fashion. The worms did not respond to tactile stimulation, and compared with wild-type worms they were sluggish and uncoordinated in both forward and backward movement. The worms moved significantly slower than wild-type worms and also had long pauses during which no movement occurred. Thus, we were not able to reliably determine changes in the direction of movement. These data indicate that normal forward and backward movement is not possible without the set of neurons that express GLR-1 but also that worms that lack these neurons are still capable of limited sinusoidal locomotion. In addition, the *glr-1::ICE* worms could switch between forward and backward movement, suggesting that other mechanisms, either neuronal or muscular, can control movement reversals.

Selective Loss of Command Interneurons Results in Defective Control of Movement

In separate experiments, we have determined that a 5 kb genomic region that is upstream of a putative NMDA receptor in *C. elegans* (*nmr-1*) drives GFP expression in a subset of the *glr-1*-expressing neurons (P. J. B. et al., unpublished data). GLR-1 is expressed in a number

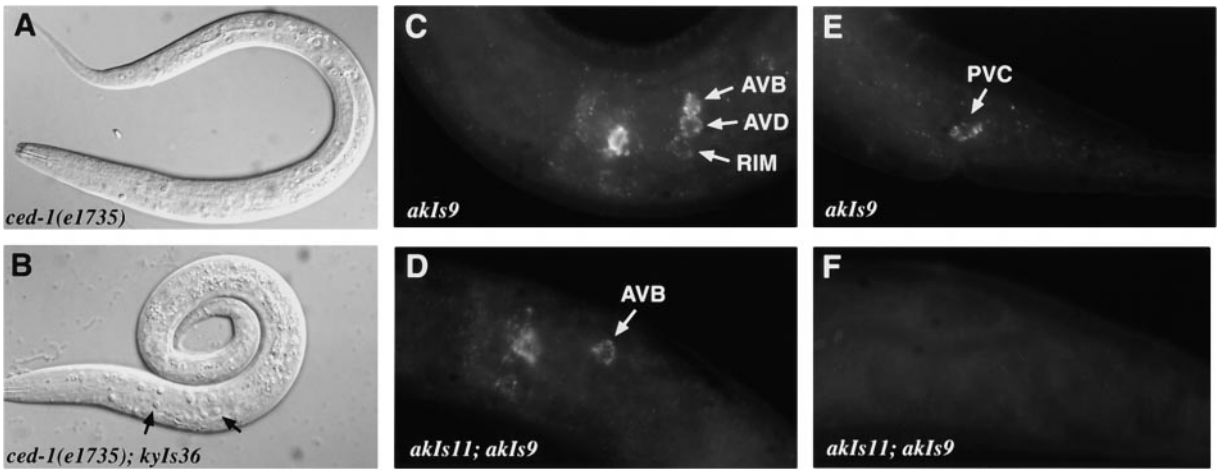

Figure 5. Expression of Mammalian ICE in Transgenic Strains Causes Selective Neuronal Deaths

(A and B) The neurons that express *glr-1* were induced to die an apoptotic death in a transgenic strain that expresses the human caspase ICE under the control of the *glr-1* promoter.

(A) A *ced-1(e1735)* worm viewed with Nomarski optics. The *ced-1* mutation prevents the normal engulfment of cells that undergo programmed cell death (Horvitz et al., 1994). A solitary apoptotic body is visible in the young larval worm.

(B) When ICE is expressed under the control of the *glr-1* promoter (*kyIs36*), many more apoptotic bodies can be observed (arrows). These correspond to the command interneurons and other neurons that express GLR-1. The death of these neurons caused the worms to be uncoordinated.

(C–F) More limited neuronal death was achieved by using the *nmr-1* promoter to drive expression of ICE in the neurons AVA, AVE, AVD, RIM, AVG, and PVC. The strain *akIs11* contains an integrated array that expresses the *nmr-1::ICE* construct. An affinity-purified polyclonal antibody directed against an extracellular epitope of GLR-1(A/T) stains the neurons AVB, RIM, and AVD (C), in addition to PVC (E), in transgenic *glr-1*::*GLR-1(A/T)* worms. AVA and AVE in the head, as well as other *glr-1*-expressing neurons, are stained but are out of the plane of focus. PVQ in the tail is very faintly stained and often not visible. Under our antibody staining conditions, neuronal staining is observed only in overexpressing strains. In the double transgenic *nmr-1::ICE;glr-1::GLR-1(A/T)*, the ICE expression causes neuronal death. In the head, AVD and RIM (D), and in the tail, PVC (F), are missing. Note that AVB is still present. Not shown are the missing AVA and AVE neurons.

of interneurons and motor neurons, including AVA, AVB, AVD, AVE, PVC, AIB, RMD, RIM, SMD, AVG, PVQ, and URY (Hart et al., 1995; Maricq et al., 1995). Compared with GLR-1, transgenic worms that expressed *nmr-1::GFP* showed a more limited GFP expression that included AVA, AVE, AVD, PVC, RIM, and AVG. All of these neurons also express GLR-1. With respect to the command interneurons, the main difference between the two promoters is that the *nmr-1* promoter does not drive GFP expression in AVB, a "forward" command interneuron. We used the same *nmr-1* promoter to drive the expression of ICE in transgenic worms. *nmr-1::ICE* (*akIs11*) worms were similar to *glr-1::ICE* worms in that they did not respond to tactile stimulation. Although they could move sinusoidally forward, their backward movement was slow and uncoordinated. In contrast to *glr-1::ICE* worms, *nmr-1::ICE* worms moved forward in a more coordinated fashion, with no pauses in movement. Their movement was also more strongly biased to move forward than that of wild-type worms, with an average duration of 113 s (Figure 6). *nmr-1::ICE* worms could still reverse and move backward, suggesting that AVB

Table 1. Analysis of ICE-Induced Neuronal Death

GFP Expression					
Stage	L1	L2	L3	L4	Adult
% GFP$^+$	99.5 ± 0.5	25.8 ± 4.6	20.5 ± 2.1	21.2 ± 2.5	22.1 ± 1.4
GFP level	+++	+++/++	++/+	+/−	+/−

Touch Response (Adults)			GFP Expression (Number of Cells)			
Anterior	Posterior	% Worms	AVA	AVE	AVD	PVC
+	+	0	—	—	—	—
+	−	20	0	0	1	0
−	+	50	0	0	0	1–2
−	−	30	0	0	0	0

Neuronal death was evaluated in a transgenic strain that expressed GFP and ICE under control of the *nmr-1* promoter (*akIs3*; *akIs11*). GFP expression was evaluated in 20 randomly chosen worms from each larval stage and first day adults. %GFP$^+$ indicates the fraction of *nmr-1::GFP*-expressing neurons that are detectable by fluorescence at a given larval stage. Body touch response was assayed in 20 randomly chosen first day adults as previously described (Chalfie et al., 1985). In worms that showed anterior touch sensitivity, faint expression could be observed in one of the two AVDs. In worms that showed posterior touch sensitivity, faint expression could be observed in one or both PVCs. +++, GFP brightness same as in *akIs3* alone; ++, dim GFP; +, very dim GFP; and +/−, barely detectable. Errors represent SEM.

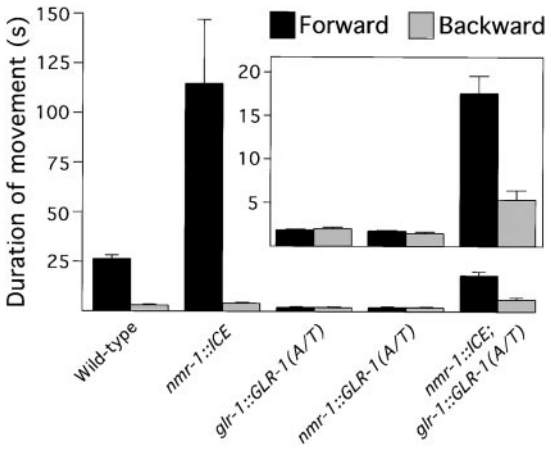

Figure 6. Expression of GLR-1(A/T) in the Command Interneurons Is Required for the Hyperreversal Phenotype

In *nmr-1::ICE* worms, all of the command interneurons except AVB undergo an apoptotic death. These worms move forward far longer than wild-type controls, 113 s ($\pm$ 33 s, n = 17, $p < 0.02$). When the *nmr-1* promoter was used to activate command neurons by the directed expression of GLR-1(A/T) (*nmr-1::GLR-1[A/T]*), the worm's forward and backward durations were similar to those of *glr-1::GLR-1(A/T)* worms, 1.65 s ($\pm$ 0.1 s, n = 8) and 1.36 s ($\pm$ 0.2 s, n = 8, $p < 0.03$), respectively (see inset), even though GLR-1(A/T) was not expressed in the neuron AVB. In the double transgenic *nmr-1::ICE;glr-1::GLR-1(A/T)*, GLR-1(A/T) is expressed in AVB—the only remaining command interneuron. Compared with *nmr-1::ICE* worms, they moved forward a shorter duration (17.3 $\pm$ 2.1 s, n = 5, $p < 0.02$). However, compared with *glr-1::GLR-1(A/T)* worms, their forward duration was increased ($p < 0.01$). Their backward movement was also increased (5.3 $\pm$ 1.1 s, n = 5, $p < 0.05$). (Inset) Expanded scale for indicated strains.

activity intrinsically cycles or that other neurons, perhaps PVD (Wicks et al., 1996), may act to limit AVB activation. When AVB was laser ablated in *nmr-1::ICE* worms, the worms no longer moved forward for long durations, and their movement became indistinguishable from that of *glr-1::ICE* worms (data not shown). As observed with *glr-1::ICE* worms, some crude circuitry for backward motion must exist in the absence of AVA, AVD, and AVE.

Expressing GLR-1(A/T) in a Subset of Command Interneurons Is Sufficient to Cause the Hyperreversal Phenotype

Our genetic ablation experiments suggested that the neurons AVA, AVE, AVD, AVB, and PVC are required for both coordinated movement and the normal rate of switching between forward and backward locomotion. The hyperreversal phenotype of *glr-1::GLR-1(A/T)* worms presumably reflects increased depolarization of the command interneurons, which changes the timing of locomotion. In which of the command interneurons is this increased activity required? To partially address the question of sufficiency, we generated *nmr-1::GLR-1 (A/T)* transgenic strains in which GLR-1(A/T) was expressed under control of the *nmr-1* promoter (*akEx52*). In these strains, expression was limited to AVA, AVD, AVE, RIM, AVG, and PVC and did not occur in AVB (see Figures 5D and 5F). In *nmr-1::GLR-1(A/T)* worms, we observed a hyperreversal phenotype similar to that described above for *glr-1::GLR-1(A/T)* worms. The average forward and backward durations were 1.65 s and 1.36 s, respectively (Figure 6). These data indicate that expression of GLR-1(A/T) in a limited subset of neurons that does not include AVB (or other GLR-1-expressing neurons, such as RMD and SMD) is sufficient to achieve a decrease in average forward duration.

In a double transgenic strain, *nmr-1::ICE;glr-1::GLR-1 (A/T)* (*akIs11;akIs9*), all of the command interneurons except AVB were killed, and GLR-1(A/T) was expressed in AVB. Using a GLR-1-specific polyclonal antibody, we examined transgenic worms that overexpressed GLR-1(A/T). Specific staining of the neurons could be clearly observed (Figures 5C and 5E). In the double transgenic strain, expression of ICE under the control of the *nmr-1* promoter killed only a subset of these neurons (Figures 5D and 5F), leaving AVB intact and expressing GLR-1(A/T). These transgenic worms moved forward for a shorter duration than *nmr-1::ICE* worms (17.3 s). However, compared with *glr-1::GLR-1(A/T)* worms, their forward duration was increased. Interestingly, their backward movement was also increased (5.3 s) (Figure 6), indicating that AVB or other GLR-1-expressing neurons may contribute to both forward and backward locomotion. When we ablate AVB in *nmr-1::ICE;glr-1::GLR-1(A/T)* worms, the behavior becomes uncoordinated and indistinguishable from *glr-1::ICE* worms. In summary, our data show that GLR-1(A/T) can be expressed in a subset of the command interneurons to cause the hyperreversal phenotype.

The Command Interneuron Circuitry Acts as a Distributed Switch that Directs Forward and Backward States

A coordinated hyperreversal phenotype is observed in both *glr-1::GLR-1(A/T)* and *nmr-1::GLR-1(A/T)* transgenic worms, suggesting that activation of a subset of the command interneurons is sufficient to change the timing of both forward and backward movements. Furthermore, *nmr-1::ICE;glr-1::GLR-1(A/T)* worms were expected to move forward for at least as long as *nmr-1::ICE* worms. Instead, their movement suggested that either AVB does not function exclusively in the forward circuitry or that other neurons contribute to the control of movement duration. These data suggest that the control of these movements cannot simply be dissected into forward and backward components. To test whether control may be partly distributed, and to further examine which neurons are required for the hyperreversal phenotype, we bilaterally laser ablated command interneurons in wild-type and *glr-1::GLR-1(A/T)* worms. Previous experiments have shown that PVC is required for the forward escape response to posterior body touch (Chalfie et al., 1985). Ablation of PVC or AVB in wild-type worms resulted in an expected decrease in the average duration of forward movement, consistent with a role for these neurons in establishing or maintaining forward movement (Chalfie et al., 1985) (Figure 7). However, similar changes in forward duration (that were not expected for a simple, nondistributed model) were observed in wild-type worms in which we ablated either AVA (backward command interneuron) (Figure 7) or the motor neuron RIM.

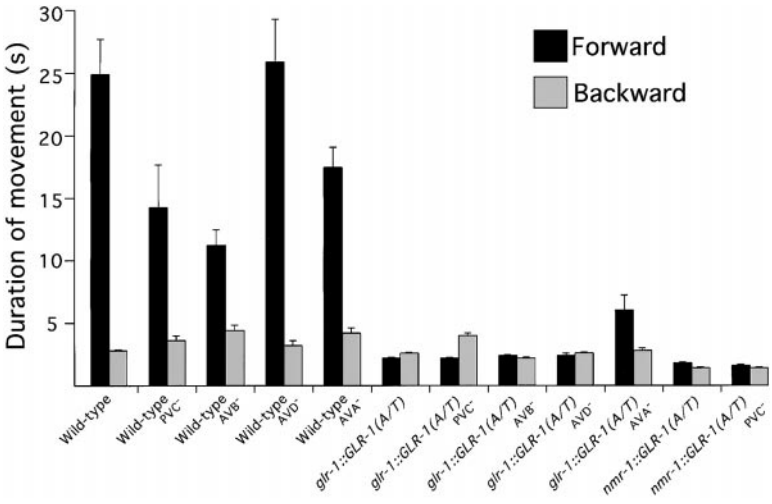

Figure 7. Ablation of Interneurons in Wild-Type and *glr-1::GLR-1(A/T)* Strains Reveals the Distributed Nature of the Circuitry Underlying Locomotory Reversals

Individual pairs of neurons were bilaterally ablated in the larval L1 stage with a focused laser beam (Bargmann and Avery, 1995). Neuronal killing had relatively little effect on reversal frequency. Wild-type worms in which PVC or AVB was ablated by laser had a decreased duration of forward motion and a slight increase in the duration of backward movement. PVC ablation in *glr-1::GLR-1(A/T)* worms caused an increase in the duration of backward movement (3.8 ± 0.4 s, $n = 6$, $p < 0.001$), whereas ablation of AVB had no effect. Ablation of AVD in either wild-type or *glr-1::GLR-1(A/T)* worms had little effect on backward or forward duration. Ablation of AVA in *glr-1::GLR-1(A/T)* worms resulted in an increase in the duration of forward movement (6.0 ± 1.3 s, $n = 6$, $p < 0.03$).

We ablated RIM because it has a unique position in the command circuitry—it receives gap junction inputs from AVA and provides synaptic inputs to AVB. Thus, it may link activation of AVA to suppression of AVB. RIM-ablated wild-type worms showed a significant decrease in the duration of their forward motion (16.9 ± 1.0 s, $n = 6$, $p < 0.02$). Moreover, these worms showed a reduced backing response to anterior tactile stimulation. Thus, RIM may have a role in both forward and backward locomotion. Ablation of AVD had no appreciable effect on duration of movement, suggesting that the primary role of AVD is to initiate backward movement in response to tactile stimuli and that AVD is not crucial for the underlying pattern generator (Chalfie et al., 1985).

When we examined the effects of ablating command interneurons in *glr-1::GLR-1(A/T)* worms, ablation of single pairs of interneurons had, on the whole, surprisingly little effect. The biggest changes were observed with ablation of AVA and PVC (Figure 7). Ablation of PVC had little effect on the duration of forward movement and increased the duration of backward movement (Figure 7), suggesting that PVC functions to limit the duration of backward movement in *glr-1::GLR-1(A/T)* worms. Ablation of AVA (a backward command interneuron) increased the duration of forward movement to 6.0 s. This is consistent with the hypothesis that activation of AVA limits the duration of forward movement. In the *nmr-1::GLR-1(A/T)* strain, GLR-1(A/T) is expressed in all of the command interneurons except AVB. Ablation of PVC in this strain did not affect the duration of forward or backward movement. This result suggests that activation of AVB (or other GLR-1-expressing neurons) in *glr-1::GLR-1(A/T)* worms contributes to backward movement. Furthermore, worms in which AVB was activated in the absence of any other command interneurons (*nmr-1::ICE;glr-1::GLR-1[A/T]*) (Figure 6) show an increase in forward duration but also a paradoxically increased duration of backward movement. In summary, we were unable to dissect the circuit into exclusive forward and backward components. At least some of the neurons, e.g., AVB, may participate in the control of both movements, suggesting that the control circuitry is in part distributed.

Sensory Input Affects the Command Interneuron Circuitry in *glr-1::GLR-1(A/T)* Worms

Although *glr-1::GLR-1(A/T)* worms were not capable of normal forward movement, they could still respond to environmental stimuli and modify their direction of movement. In wild-type worms, touch to the body or nose elicits an escape response, indicating that sensory input can interrupt the intrinsic pattern generator. Appropriate escape responses to tactile stimuli were also observed in *glr-1::GLR-1(A/T)* worms, indicating that the hyperreversal phenotype did not preclude modulation by sensory input (data not shown). Another example of sensory input overriding the default movement program was observed in the response to volatile repellants, such as octanol (Troemel et al., 1995). Both wild-type and *glr-1::GLR-1(A/T)* worms backed away from a sharp stick, coated with octanol, that was placed near the nose of the worm (data not shown). *glr-1::GLR-1(A/T)* worms could be made to back up continuously when the octanol-coated stick was constantly maintained in front of the worm as it backed away. These data indicate that sensory processing and the sensory control of locomotion can still take place in a hyperreversing *glr-1::GLR-1(A/T)* worm.

Activation of ASH by GLR-1(A/T) Reduces the Duration of Forward Movement

Specialized neuronal processes that extend to the tip of the worm's nose are mechanosensitive and allow a worm to detect an object in its path. Detection initiates an avoidance response; the worm reverses direction and moves backward for several body lengths. The polymodal sensory neuron ASH (Figure 1) is required for the efficient execution of this avoidance response (Kaplan and Horvitz, 1993). How ASH signals to the command interneurons is unknown. There are two likely hypotheses: (1) mechanical stimulation causes a graded depolarization and a consequent increase in transmitter release or (2) mechanical stimulation causes a graded hyperpolarization of the neuron and a consequent decrease of synaptic transmitter release—as is observed in the signaling of light by vertebrate photoreceptors.

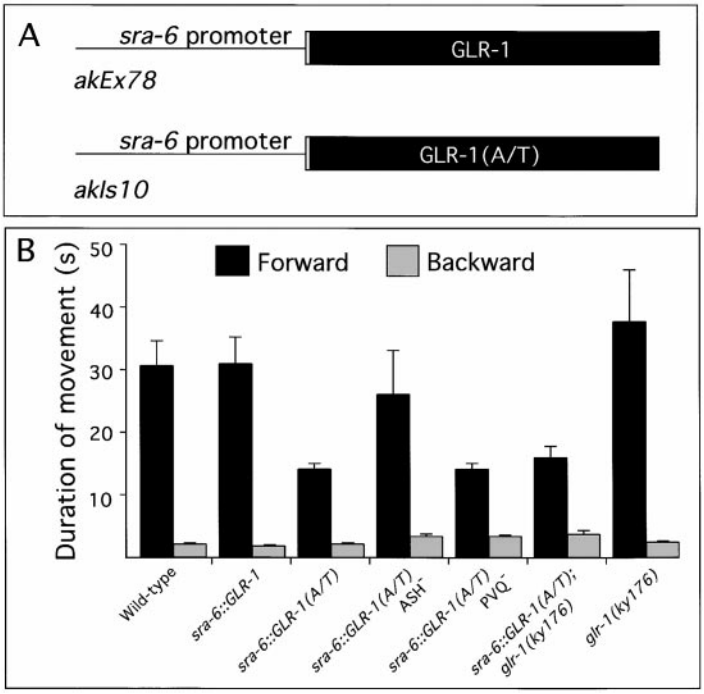

Figure 8. Ectopic Expression of GLR-1(A/T) in the Sensory Neuron ASH Increases the Number of Reversals and Decreases the Duration of Forward Locomotion

(A) The *sra-6* promoter (Troemel et al., 1995) was fused in-frame to a 5 kb genomic clone encoding full-length GLR-1 (*akEx78*) or GLR-1 (A/T) (*akIs10*). This promoter drives GFP expression in the neurons ASH and PVQ, and weakly in ASI.

(B) ASH provides synaptic input to the command interneurons and is required for the avoidance of tactile stimuli (White et al., 1986; Kaplan and Horvitz, 1993). Compared with wild-type worms or worms that overexpressed GLR-1 (*sra-6::GLR-1*) in ASH, *sra-6::GLR-1(A/T)* worms on average moved forward for a briefer duration (14 ± 1.2 s, n = 20, $p < 0.001$). In two additional independent transgenic strains that expressed *sra-6::GLR-1(A/T)*, the average forward durations were 10.34 ± 3.2 s and 10.2 ± 1.7 s. Transgenic worms in which PVQ had been laser ablated (PVQ^-) were indistinguishable from nonablated worms. The *sra-6::GLR-1 (A/T)* phenotype could be rescued by ablating ASH.

To test these possibilities (and to determine whether GLR-1(A/T) could be used to activate neurons that do not normally express GLR-1), we expressed GLR-1 (A/T) in the ASH neurons. Previous experiments have shown that GFP was expressed strongly in ASH when fused to the *sra-6* promoter (Troemel et al., 1995). Expression was also noted in two neurons involved in chemosensory signaling, PVQ and ASI (faint expression). We used the same *sra-6* promoter to drive expression of GLR-1(A/T) in ASH in the transgenic strain *akIs10* (Figure 8A). To control for specificity of expression, we included two controls. First, transgenic strains that expressed an N-terminal GFP fusion to GLR-1(A/T) under control of the *sra-6* promoter had detectable fluorescence only in ASH, PVQ, and ASI (data not shown). These worms also had a shortened duration of forward movement. Second, transgenic strains that expressed a promoterless GLR-1(A/T) construct had no detectable fluorescence (data not shown), indicating that the intronic sequences in the *glr-1* genomic clone were insufficient to drive detectable levels of protein expression in the command interneurons or any other neuronal tissue.

Compared with wild-type worms, *sra-6::GLR-1(A/T)* (*akIs10*) worms reversed direction approximately twice as frequently. The increased number of reversals was due to a reduction in the forward transit time from ~30 s in wild-type worms to 14 s (Figure 8B). We observed similar behavior in two additional independent transgenic strains that expressed *sra-6::GLR-1(A/T)*. This difference was not observed in *sra-6::GLR-1* transgenic worms that overexpressed wild-type GLR-1 in ASH (*akEx78*). The duration of backward motion in *sra-6::GLR-1(A/T)* worms was not different from that observed in wild-type worms.

Expression of GLR-1(A/T) in ASH and tactile stimuli to the nose have the same behavioral response—a decrease in the duration of forward movement. In addition, expression of GLR-1(A/T) in the command interneurons (*glr-1::GLR-1[A/T]* and *nmr-1::GLR-1[A/T]*), the synaptic targets of ASH, also reduced the duration of forward movement. These results suggest that ASH is depolarized by mechanical stimuli, and this increases excitatory synaptic transmission to the command interneurons. Interestingly, because no putative glutamate receptors are believed to be expressed in ASH (P. J. B. et al., unpublished data), these results provide additional evidence that GLR-1(A/T) can function as a homomeric receptor in the worm. There is no evidence that the chemosensory interneuron PVQ has a major role in the control of locomotion, and, anatomically, there are no direct synaptic connections with the command interneurons. However, because *glr-1* is normally expressed in PVQ, we laser ablated PVQ to confirm that the increased reversals observed in *sra-6::GLR-1(A/T)* worms were due to activation of ASH. Transgenic worms in which PVQ had been bilaterally laser ablated (*sra-6::GLR-1(A/T)*; PVQ^-) were indistinguishable from nonablated worms (Figure 8). In contrast, bilateral ablation of ASH in *sra-6::GLR-1(A/T)* worms reversed the effect of GLR-1(A/T) expression by the *sra-6* promoter. Interestingly, the duration of movements in worms that expressed *sra-6::GLR-1(A/T)* was not affected by a null mutation in the *glr-1* gene. The movement of the *sra-6::GLR-1(A/T);glr-1(ky176)* strain was similar to that observed in *sra-6::GLR-1(A/T)* worms alone, suggesting that synaptic transfer occurs in the absence of the endogenous *glr-1* gene product.

Discussion

The molecular and cellular basis for locomotion is not well understood in any system. As we have shown, *C. elegans* provides many advantages for a genetic based

study of the neural circuitry that controls movement. Elucidating the neuronal control of worm locomotion is also important for an understanding of many more complicated behaviors, such as chemotaxis and thermotaxis. Simple pattern generators are not exclusive to the worm's nervous system. It is likely that all nervous systems are made up of increasingly complicated arrangements of local circuit building blocks. In the case of the worm, five pairs of interneurons are known to be required for coordinated backward and forward movement (Chalfie et al., 1985). The control of this movement is important for foraging. In the presence of food, the worm is likely to remain near the food; in the absence of food, the worm must change its strategy and move about in search of food. Presumably, foraging strategies can be optimized for particular environments, and the underlying neuronal circuitry exhibits some degree of plasticity (Wicks and Rankin, 1995).

To better understand how simple neuronal circuits encode information, we have generated transgenic strains that express, under control of neural specific promoters, a modified GLR-1 receptor that contains the Lurcher A/T mutation (Zuo et al., 1997). These transgenic strains display a dramatic change in foraging behavior that is consistent with a transgene-dependent neuronal activation. We have shown that this perturbation approach can be of general use: it works in neurons that normally express glutamate receptors as well as in neurons that are not known to express glutamate receptors. Our examination of the timing behavior of *C. elegans* is an example of using a genetic technique to introduce a modified cation-selective channel predicted to depolarize neurons in transgenic animals.

To address the contribution of specific neurons to circuit function, we expressed the human caspase ICE in transgenic worms and showed that we were able to effect an apoptotic death of selected neurons. In worms, *ced-3* encodes a caspase that acts to induce programmed cell death (Yuan et al., 1993), and overexpression of *ced-3* can kill cells in *C. elegans* that normally live (Shaham and Horvitz, 1996). Overexpression of *ced-3* leads to incomplete cell death (Shaham and Horvitz, 1996) due to the protective action of *ced-9* (Hengartner and Horvitz, 1994). Reasoning that *ced-9* might be less effective in regulating the activity of a mammalian caspase, we chose to overexpress human ICE in the *C. elegans* nervous system (Cerretti et al., 1992; Thornberry et al., 1992). We have not compared ICE with CED-3, but our results indicate that overexpression of the mammalian ICE is an effective way of genetically ablating neurons which may be an improvement over techniques that cause a necrotic death of neurons (Maricq et al., 1995).

When expressed in the command interneurons (*glr-1::GLR-1[A/T]* and *nmr-1::GLR-1[A/T]* worms), GLR-1 (A/T) increases reversal frequency by decreasing the duration of forward movement. The GLR-1(A/T) pore becomes less leaky and less permeant when the glutamine that lines the pore is mutated to arginine. If GLR-1(A/T;Q/R) is expressed in the command interneurons (*glr-1::GLR-1[A/T;Q/R]*), the change in reversal frequency is less dramatic. This effect is consistent with the decreased current observed when GLR-1(A/T;Q/R) rather than GLR-1(A/T) is expressed in *Xenopus* oocytes. Although we suggest that GLR-1(A/T) depolarizes the command interneurons and that this depolarization leads directly to the observed behavioral changes, it may be that secondary, more complicated processes, such as adaptation, contribute to the hyperreversal phenotype. In mice, the Lurcher mutation induces apoptotic death of Purkinje neurons (Zuo et al., 1997). Perhaps neurons in the worm are less readily damaged by depolarization or calcium influx, or GLR-1(A/T) is less permeant.

In the simplest model, depolarization of the forward command interneurons activates forward motion and simultaneously inactivates the backward command interneurons—presumably by hyperpolarization mediated by an unidentified neurotransmitter. Limited evidence for this model was provided by serial electron microscopic sections that revealed the presence of reciprocal synaptic contacts between the forward and backward command interneurons (Chalfie et al., 1985; White et al., 1986) (Figure 1). Neuronal input from sensory receptors would be expected to bias this bistable circuit by altering the dwell time in either the forward or backward state (Chalfie et al., 1985; Wicks et al., 1996; Lee et al., 1999).

We used a genetic approach to determine whether the perturbation of the command interneurons was the proximal cause of the hyperreversal movement defect of *glr-1::GLR-1(A/T)* worms or whether these perturbations required functional sensory neurons to achieve the movement defect. A variety of mutants that affect the birth, differentiation, or function of the body wall touch receptors (Way and Chalfie, 1988; Driscoll and Chalfie, 1991; Xue et al., 1992) did not influence the movement disorder, suggesting that this circuit can control the direction of movement without input from these sensory neurons.

Our findings suggest that the frequency of movement reversals is an intrinsic property of the command interneurons and that reversal frequency is modulated by the level of neuronal depolarization. Based on our genetic studies, we propose the following model for the control of reversal frequency (Figure 1). A forward circuit that contains the forward command interneurons (PVC and AVB), and perhaps other neurons, is generally active and drives forward locomotion. Because the activity is already relatively high, the additional conductance provided by the GLR-1(A/T) channels may not have much effect on membrane depolarization. On the other hand, expression of GLR-1(A/T) in the normally less active backward circuit that contains the backward command interneurons (AVA, AVE, and AVD), and perhaps other neurons, would tend to cause a bigger change in activity. In the case of *glr-1::GLR-1(A/T)* worms, all of the command interneurons express GLR-1(A/T). However, the phenotype of *nmr-1::GLR-1(A/T)* worms (no expression of GLR-1(A/T) in AVB), either with or without PVC ablated, was quite similar to that of *glr-1::GLR-1(A/T)* worms, strongly suggesting that the hyperreversal phenotype was most dependent on expression of GLR-1 (A/T) in the backward command interneurons.

When the forward circuit is active, the model predicts that it inactivates the backward circuit via an inhibitory synaptic connection. However, the increased activity of the backward command interneurons in *glr-1::GLR-1*

(A/T) worms will quickly counteract the synaptic inhibition, resulting in the activation of the backward command interneurons. This activation, in turn, will inactivate the forward circuit via an inhibitory synaptic connection. Thus, the worm rapidly moves backward and forward. This inhibition has been predicted in modeling studies (Wicks et al., 1996). The model assumes that GLR-1(A/T) increases the switching frequency by increasing the rate of neuronal depolarization and the subsequent rise in intracellular Ca^{2+} concentration. Alternatively, GLR-1(A/T) may simply affect basal Ca^{2+} levels.

Our results suggest that neurons in the locomotory control circuitry cannot be strictly categorized as forward or backward. Some neurons appear to be active in both responses. This type of circuit, where neurons are not strictly assigned a single function but rather can be active in several, even opposing, behaviors, is characteristic of distributed neuronal circuits and is thought to increase the behavioral repertoire of small nervous systems. Distributed information processing has been described by Kristan and coworkers, particularly for the interneuronal network that produces bending in the leech (Kristan et al., 1995).

We have shown that ablation of any one interneuron in *glr-1::GLR-1(A/T)* worms has relatively little effect on the duration of forward or backward movement, and ablation of some neurons appears to have opposite effects, depending on the activity of the rest of the circuit. Additional evidence for distributed function can be seen in the mosaic analysis performed by Kaplan and coworkers (Hart et al., 1995). They showed that the worm's backing response to tactile stimulation of the nose required that GLR-1 be expressed in AVB (and perhaps PVC)—the forward command interneurons.

Interestingly, worms can still move backward and forward, although they are slow and uncoordinated, even when all of the command interneurons have been killed, indicating the existence of additional gating mechanisms. These secondary gating functions may be located in additional interneurons, in the motor neuron circuitry that controls muscle contraction or in the muscles themselves.

Although not required for the hyperreversal phenotype, input from sensory neurons influences the relative duration of backward and forward movement. For example, the forward movement of *glr-1::GLR-1(A/T)* worms could be interrupted by anterior body touch, causing the worms to move backward. Modification of the reversal frequency suggests that the command interneurons integrate a variety of sensory inputs to determine the final direction of movement. Expression of GLR-1(A/T) in the sensory neuron ASH biases the worm to move forward for shorter durations of time. Presumably, this is secondary to the depolarization of ASH, which leads to an increase in the probability of neurotransmitter release. How neurotransmitter release is coupled to activation of the backward command circuitry is unclear. It is not dependent on *glr-1*. Sensory input clearly modifies the behavior of *glr-1::GLR-1(A/T)* worms, e.g., touch, volatile repellants, food, and dauer state all affect locomotion.

We have shown that the pattern of locomotion changes dramatically in transgenic strains that express a dominantly active form of GLR-1 in the locomotory control neurons. Our findings suggest a strategy for uncovering gene products required for the proper function of the locomotory control circuitry. Mutations that modify the hyperreversal phenotype of the transgenic *glr-1::GLR-1(A/T)* worms may affect genes that influence intrinsic neuronal properties and synaptic signaling.

Experimental Procedures

General Methods and Strains

Nematodes were grown at 20°C under standard conditions that included uncrowded conditions and the presence of ample food—the *E. coli* strain OP50 (Brenner, 1974). Wild-type nematodes were *C. elegans* strain N2. Functional studies of GLR-1 and variants were conducted by heterologous expression in *Xenopus* oocytes. cRNAs encoding GLR-1, GLR-1(A/T), and GLR-1(A/T;Q/R) were injected into *Xenopus* oocytes, and currents were measured with standard two-electrode voltage-clamp recording techniques (Maricq et al., 1991).

Expression Constructs

Promoter fusions for the gene *glr-1* and changes in the *glr-1* coding region were generated by standard molecular biological techniques. pV1: a 10.7 kb PstI–XhoI genomic DNA fragment containing the *glr-1* gene was subcloned into the Bluescript SK(+) vector (Stratagene). pYZ8: by using a PCR-mediated mutagenesis approach, the A/T change found in the δ2 ionotropic glutamate receptor of Lurcher mice was engineered into pV1. pPB32: a 5 kb *nmr-1* promoter (F07F6) inserted into pYZ8 replaced the *glr-1* promoter. pYZ25: the Q/R change found in TM II of certain subtypes of ionotropic glutamate receptors was introduced into pYZ8 by PCR mutagenesis. PCR products were amplified with the high-fidelity Pfu taq polymerase (Stratagene), using the standard conditions recommended by the manufacturer. The PCR-amplified region was resequenced to confirm that no additional changes were introduced into *glr-1*. pYZ13: the *glr-1* promoter region (BamHI–SalI fragment) of pV1 was excised and replaced with the *sra-6* promoter (BamHI–SalI fragment). The sequence of the chimeric constructs was confirmed by restriction enzyme analysis and DNA sequencing of the junctional regions. pV32: the full-length cDNA encoding ICE (Cerretti et al., 1992) was engineered into multiple cloning site II (MCS II) of the expression vector pPD49.26 (Fire et al., 1990), and the *glr-1* promoter region (BamHI–SalI fragment) of pV1 was engineered into MCS I. pPB14: the *glr-1* promoter region of pV32 was replaced by a 5 kb PCR product upstream of the predicted F07F6.6 (*nmr-1*) gene product. pPB24: an N-terminal GFP fusion to GLR-1 was constructed by cloning the *glr-1* promoter region of pV1 (extending through the putative signal sequence) into MCS I and MCS II of the GFP expression vector pPD114.108 (a generous gift of Andy Fire). The coding region of *glr-1* was subcloned in-frame into MCS III and MCS IV of pPD114.108.

Transgenic Strains

All transgenic strains were generated by the standard technique of microinjection to achieve germline transformation (Mello et al., 1991). In all cases, *lin−15(n765ts)* worms were injected with the *lin-15* rescuing plasmid (pJM23, 30 μg/ml), along with one of the following plasmids (pV1, pYZ8, pYZ25, pYZ13, pPB14, pPB32, or pPB24, each at 70 μg/ml). Multiple independent extragenic lines were generated for each transgenic strain. These lines were readily identified by the rescue of the *lin-15* multivulva (Muv) phenotype. Transgenes were chromosomally integrated by psoralen mutagenesis and repeatedly outcrossed (Yandell et al., 1994). The phenotype of the integrated lines did not differ from the behavior of rescued non-Muv worms in the extragenic lines.

Movement Assays

Worms were grown at 20°C–21°C on *E. coli* strain OP50 under standard conditions (Brenner, 1974). Single first day adult worms were transferred by glass pick from growth plates to standard agar plates with no food. Care was taken to ensure that the worms were free of adherent bacteria and that no bacteria were transferred with the

worm. Each worm was observed for ~7 min, and a computer program keyed by the operator recorded the duration of the worm's forward and backward movements. All movement assays were scored blind. Forward and backward durations reported throughout the text are mean ± SEM. Average duration times varied between experiments, presumably due to variables such as humidity, dryness of plates, etc. For this reason, for a given experiment all of the strains that needed to be scored were randomized and scored on the same day. Worm velocity was determined from calibrated, videotaped recordings of worm movements on unseeded agar plates. Average velocity was defined as the total path distance (mm) per time (s). Reversal frequency was obtained by counting the total number of movement reversals per minute; it can be calculated from the forward and backward duration: reversal frequency per min = 1/([forward time + backward time]/2).

Laser Ablation of Neurons

Laser ablation of neurons was achieved, using standard protocols (Avery and Horvitz, 1987; Bargmann and Avery, 1995). To help identify neurons, we used an unpublished transgenic strain (*akIs3*) that expresses the reporter molecule GFP (Chalfie et al., 1994) in most of the command interneurons. These strains were indistinguishable from wild-type with respect to forward and backward movement time. The double transgenic integrant (*akIs9;akIs3*) was indistinguishable from *akIs9*. Laser kills were confirmed by direct examination for the loss of GFP fluorescence and by behavioral assay. For example, PVC-ablated worms had characteristic defects in tail touch response, and sensory neuron ablations render the worm touch insensitive (Chalfie et al., 1985).

Antibody Staining and Microscopy

A GST fusion gene was constructed by inserting a cDNA encoding a 22 amino acid fragment of the extracellular domain of GLR-1 into the pGEX vector (AMRAD). A fusion protein of the predicted size was purified on GST agarose beads and injected subcutaneously into rabbits, using standard protocols (Harlow and Lane, 1988). Antibody experiments used affinity-purified polyclonal sera purified against maltose binding protein (NEB) fused in-frame to the same 22 amino acid fragment of GLR-1. On Western blots prepared from wild-type worms, the polyclonal sera could detect a 97 kDa band that is predicted to be the *glr-1* gene product. This band is missing in protein extracts prepared from a strain, *glr-1(ky176)*, that contains a deletion mutation which lacks the 22 amino acid epitope.

To detect GLR-1(A/T) expression in integrated transgenic strains, we prepared worms according to the fixation, permeabilization, and staining protocols described by Finney and Ruvkun (Finney and Ruvkun, 1990). Primary antibodies were used at a dilution of 1:100, and an Oregon Green–conjugated goat anti-rabbit secondary antibody was used at 1:200 (Molecular Probes). Epifluorescence images were acquired with a Zeiss Axioskop microscope and a Princeton Instruments Micromax charge-coupled device camera. Confocal images were acquired with an Optiphot-2 microscope (Nikon) and a BioRad Confocal Imaging System.

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